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FINAL TECHNICAL
REPORT

SUBMITTED

BY

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Name of Institution: The Florida Agricultural and
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School of Pharmacy,
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Title of Investigation: Microbial Transformation of
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Principal Investigator: Dr. S. S. Lamba

NARRATIVE:

The study involving the use of coulter counter in studying the effects of Neomycin on E. coli, S. aureus and A. aerogenes was completed. The purpose of this was to establish proper technique for enumeration of cells per ml. It was found that the inhibitory effects on growth of E. coli and A. aerogenes, both gram negative organisms were directly related to the concentration of neomycin used. However, in case of S. aureus, a gram positive organism, a decreased inhibition was noted at higher concentrations. A paper entitled, "Use of Coulter Counter in studying effect of drugs on cells in culture I. Effects of neomycin on E. coli, S. aureus and A. aerogenes" was published in "Phyton", 34 (2), 13-16, 1976. A copy of this paper is attached in appendix I.

Laboratory procedures were also established to study the effects of nucleoside antibiotic cordycepin on He La cell (SH-503, International Scientific Industries) grown in suspension cultures. Cordycepin was one of the first nucleoside antibiotic isolated from the mold Cordyceps militaris. It has also been isolated from Aspergillus nidulus. Cordycepin is a structural analog of adenosine. It is a cytotoxic agent in which the sugar portions are pentose and cordycepose. The cells were grown as monolayer cultures on Eagle's Minimum Essential Medium. Once the cells had grown fairly thick as monolayer, they were trypsinized using 0.25 per cent trypsin and transferred to the suspension (spinner)

medium, which has the same composition as Eagle's medium except it does not contain calcium chloride but contains ten times the phosphates and also 0.1% of carboxy methyl cellulose was used to maintain the cells in suspension. Culture replicates containing 20 ml of medium were used and incubated at 38°C. An initial cell inoculum of 2.0×10^4 to 5×10^4 cells per ml as enumerated by the coulter counter was used. A total of 10 counts were made and counts averaged. The Coulter Counter duplicates itself very well, the difference in the counts fell within a 5 per cent experimental error.

The drug cordycepin is not readily soluble in water; therefore, it was dissolved in 0.2 ml of dimethyl-sulfoxide and then triple distilled water was used to make up the various concentrations used (1, 5, 10, 100, 200 and 500 $\mu\text{g/ml}$). In order to see, if dimethyl-sulfoxide affected cell growth, an amount equal to that contained in the test flasks was added to the control flasks. It was found that this did not inhibit growth.

The cultures were incubated for 5 days and counts were recorded every 24 hours. It was noted that cordycepin 100 $\mu\text{g/ml}$ incorporated at the start of the incubation period resulted in 76 per cent growth inhibition of cells for about 24 hours. It appears that exposure of cells to cordycepin results in growth inhibition for about 24 hours, at which time cell division seems to resume. This indicates that the observed growth inhibition is cytostatic rather than cytotoxic. There is a close structural similarity between cordycepin and deoxyadenosine. It is quite possible that cordycepin

may compete with adenosine for phosphorylation to the active nucleotide level. The results of this study were presented at the Fourth Annual Xavier-Minority Biomedical Symposium in April 1976. An abstract of the presentation is attached in Appendix II. Attempts were made to biotransform cordycepin by incubating it in cultures of Bacillus megaterium (ATCC 13368), but were unsuccessful. However, incubation of cordycepin with B. subtilis brought about hydrolysis and the products were identified as adenine and cordycepose.

Formycins, which are c-nucleosides were found to undergo deamination by purified adenosine deaminase, prepared from streptomyces and aspergilli. Formycin was converted to formycin B and Oxoformycin B by Nocardia interforma. This has also been reported by O.K. Sebek in Advances in Applied Microbiology, 14, 123-146 (1971).

Attempts to biotransform other nucleoside antibiotics were not successful. However, time did not permit to experiment with some other organisms, which may prove useful.

Conclusion: The project provided exposure to three students involving the following:

1. Basic microbiological techniques for preparation, filtration and sterilization of media.
2. Preparation fo glassware and apparatus for cell culture studies.

3. Use of Coulter Counter , multichannel analyzer and x-y plotter for determining frequency size distributions of cell suspensions.
4. Analysing and reporting the data obtained.
5. Basic physico-chemical techniques involving isolation of products from culture filtrates.

APPENDIX I

ΦΥΤΟΝ

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34(2): 109-116, XI-1976

Use of Coulter Counter in studying effects of drugs on cells in culture I. Effects of neomycin on *E. coli*, *S. aureus* and *A. aerogenes*¹

S. S. Lamba & D. E. Simpson²

ABSTRACT. — The application of the Coulter Counter to study antimicrobial effects of drugs is relatively new. The method measures the resistance of a conducting solution as a particle passes through an aperture. The technique used involves the counting of suitably diluted samples of microbial cultures at specific time intervals using the Coulter Counter, Channelyzer, and Plotter in determining the rates of microbial generation under varying conditions. In this paper are reported some conclusive results of the effects of the antibiotic neomycin in concentrations ranging from 1 to 10,000 $\mu\text{g/ml}$ on the growth of *E. coli*, *S. aureus*, and *A. aerogenes*. The inhibitory effects on growth rates of *E. coli* and *A. aerogenes*, both gram negative organisms, were directly related to the concentrations of neomycin used. However, in case of *S. aureus*, a gram positive organism, a decreased inhibition was noted at higher concentrations.

Coulter Counters (1, 2, 3, 4) are being employed as a new approach in the study of drugs and their effects on microorganisms. This versatile instrument affords a new dimension for making faster and more accurate total population counts of sample organisms grown under controlled conditions. This technique, as compared to the conventional technique, offers many advantages, such as speed, accuracy

¹ Presented at the American Pharmaceutical Association and Academy of Pharmaceutical Sciences (Pharmacognosy and Natural Products Section) meeting held in San Francisco, California, April 19-24, 1975. • This study was in part supported by grants from the National Institutes of Health and National Aeronautics and Space Administration. The authors are also indebted to Mrs. B. H. Appleyard for technical assistance. ² School of Pharmacy, Florida A & M University, Tallahassee, Florida 32307, U.S.A.

and simplicity. The actual counting is accomplished by a special system utilizing a mercury manometer and aperture. This system is connected to a vacuum supply which siphons the solution containing organisms through an aperture which interrupts what is known as a current path. Each organism represents a resistance in the current path which is sensed electronically when passing through the aperture. The resistance is sensed as an electrical pulse which is an input signal to an analog digital circuit which amplifies and converts each pulse into a digital representation displayed on a set of digital readout nixie tubes. Coulter Counter is a convenient tool for the determination of total cell counts (5).

Neomycin is an aminoglycoside antibiotic isolated from the *Streptomyces* species. It is mostly effective against gram negative organisms. It is one of the most commonly used topical antibiotics. It has also been used as a standard to compare some of the newer antibiotics such as kanamycin and gentamycin. It was, therefore, felt that additional data obtained on the effects of neomycin on gram negative and gram positive organisms using Coulter Counter will be useful. Such data will hopefully enable the pharmacokineticists to predict antibacterial doses necessary to administer to maintain a desired minimum biologically active concentration. In this paper are reported some conclusive results of the effects of neomycin on the growth of *Escherichia coli*, *Aerobacter aerogenes* and *Staphylococcus aureus*, using the Coulter Counter technique.

EXPERIMENTAL & METHODS. — Test organisms. — Microorganisms used in this study were *E. coli*, ATCC25922, *S. aureus* (Bact-Chek B1170-7, Roche Laboratories, New York, N.Y.) and *A. aerogenes*. (Bact-Chek Roche Laboratories, New York, N.Y.). Replicate broth solutions were seeded from stock samples. The stock cultures were allowed to grow for 24 hours before seeding of the test samples.

Culture media. — Nutrient broth and Antibiotic medium III (Difco Laboratories, Detroit, Michigan) were prepared according to the specifications of the manufacturer, then filtered three times through 0.45 millipore filters and then autoclaved at 121° C for 15 minutes. The pH of the medium was adjusted to 7.00 ± 0.1 .

Antibiotic. — An assayed sample of neomycin sulfate (Nutritional Biochemical Corporation) was used and will be referred to here as neomycin. The neomycin solutions were sterilized using 0.22 μ type Swinny type millipore filters prior to use.

Bacterial cultures. — Fifty-ml. aliquots of culture medium was inoculated with 0.5 ml. of the stock organisms which were grown in an incubator with the temperature maintained at 37.5° C. The samples were seeded with 1×10^4 organisms per sample. The samples were

allowed to grow to a cell population of 1×10^5 /ml. At this stage the organisms were in the exponential phase of growth and the drug was incorporated.

Total count method. — Aliquots of 0.2 ml. were withdrawn from each sample and placed into 20 ml. of sterile 0.85 % saline solution (Isoton [Scientific Products]). This dilution allowed a satisfactory concentration for monitoring of the growth. Total counts were recorded every 30-45 minutes until the control samples reached their stationary phase.

The instrument control settings were: aperture current 1/2; amplification 2; gain 8; matching switch 60; lower threshold 5; upper threshold, maximum, and a 50 lambda manometer with a 30 micron aperture. The above mentioned operational conditions were found to produce the best results without noticeable interference from background particles or electrical noise from the equipment used. The saline solution was filtered three times through a 0.45 micron millipore filter. The solution was then counted to determine background level. A maximum level of 50 particles per sample was obtained. This background level was insignificant when compared with the total counts

Effects of antibiotic concentration on generation. — Fresh solutions of neomycin were aseptically prepared for each segment of this experiment. The concentrations of neomycin used were 1, 10, 100, 500 and 1,000 μ g/ml. The above concentrations were added to replicate of 50 ml. samples of the organisms which were maintained at 37.5° C in the Constant-Temperature Shaker bath. The effects of various neomycin concentrations on growth rate of *S. aureus*, *E. coli*, and *A. aerogenes* were monitored by the total count method.

RESULTS & DISCUSSION. — The rate of growth of a bacterial culture at a given moment is directly proportional to the number of cells present at that moment. This relationship is given by the following equation:

$$\frac{dN}{dt} = KN$$

Integration of the above expression gives:

$$N = N_0 e^{kt}$$

where N_0 is the number of cells at time zero and N is the number of cells at any later time t , and k is growth constant. Solving the above equation for k gives:

$$k = \frac{\ln (N/N_0)}{t}$$

Thus k represents the rate at which the natural logarithm of cell number increases with time and can be determined as slopes graphically.

Effect of neomycin on growth rates. — The effects of graded concentrations of neomycin on the growth of *E. coli*, *A. aerogenes* and *S. aureus* are shown in figures 1, 2 and 3. Since the semilogarithmic plots were completely linear and showed no lag phase or induction period, in other words there was an exponentially growing culture, the following equation could be applied:

$$\log N = \frac{kt}{2.303} + \log N_0$$

where N = number of organisms/unit volume
and N_0 = cell concentration at initial time.

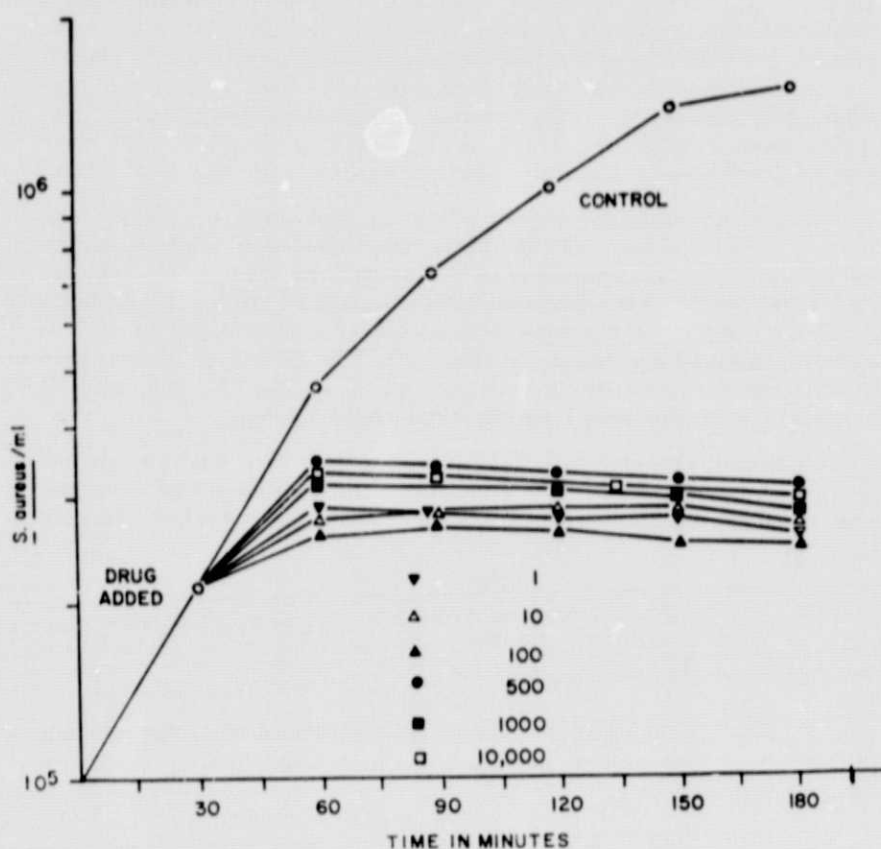


FIG. 1. Semilogarithmic plots of *E. coli*/ml. against time by total counts (Coulter) in the presence of indicated concentrations of neomycin in micrograms per milliliter.

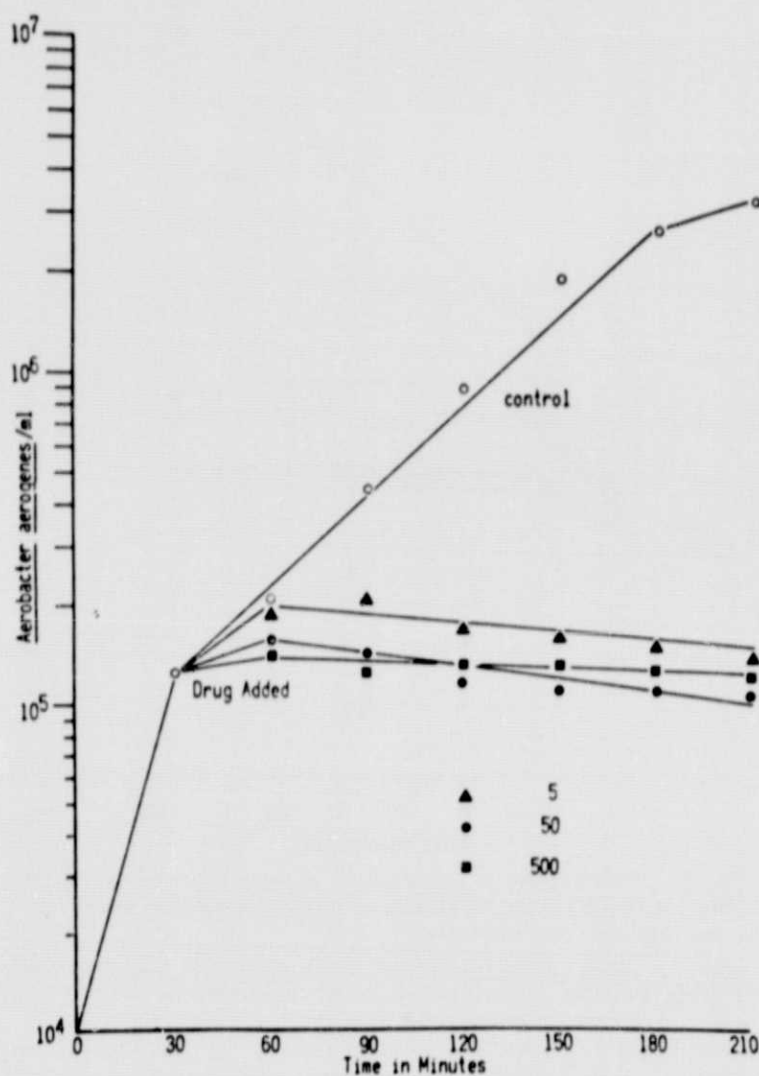


FIG. 2. Typical semilogarithmic plots of *A. aerogenes* growth by total counts in the presence of indicated concentrations of neomycin in micrograms per milliliter.

In these circumstances, any change in the net rate of generation is characterized by the constant K which can be assigned to the effect of neomycin. This can be deduced by the change in slope of the curves. From the slope of the linear portions generation constants $K_{apparent}$ were obtained. The apparent generation rate constants (K_{app}

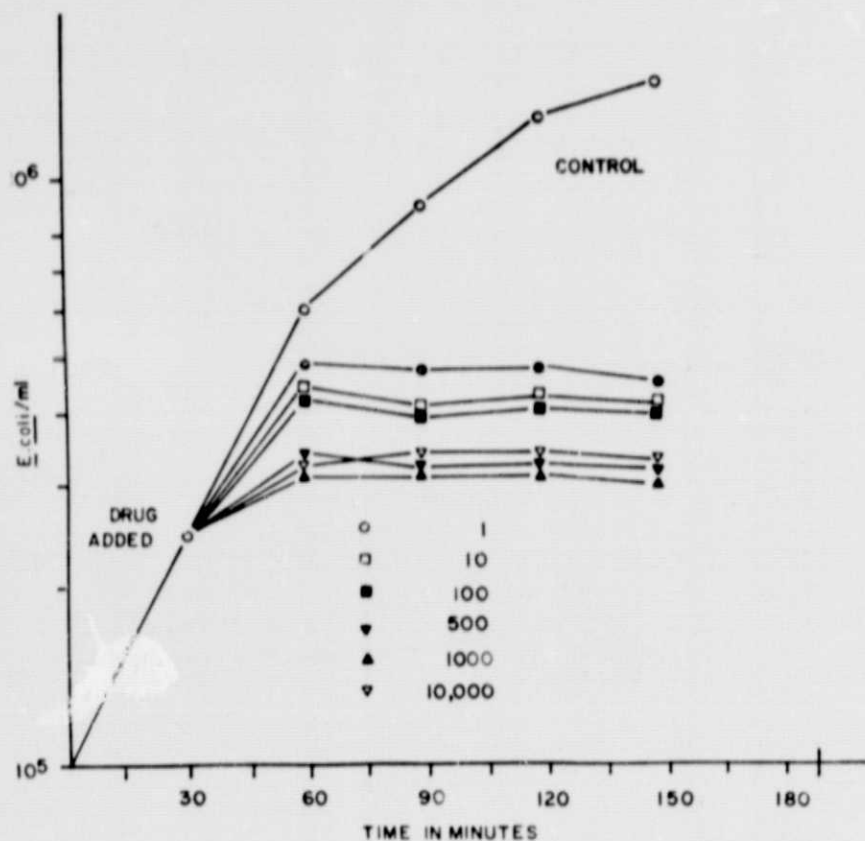


FIG. 3. Semilogarithmic plots of *S. aureus* growth by total counts in the presence of indicated concentrations of neomycin in micrograms per milliliter.

parent) were initially linearly dependent on neomycin concentrations. The K_0 and $K_{apparent}$ values for neomycin concentrations used are shown in Table I, where K_0 represents slope for the control curves and $K_{apparent}$, the slopes for the neomycin added curves.

The decreased generation rates observed in the presence of neomycin concentrations used, where $K_{apparent}$ values were still positive, must be due to the inhibition of generation rate. This has also been reported to be so for tetracycline (6), lincomycin (1), spectinomycin (7) and erythromycin (8). Many workers (9, 10, 11) have reported inhibition of protein synthesis at low antibiotic concentrations. It is believed that the cultures inhibited by neomycin grow in balanced growth and the rate of cell division is proportional to the total rate of protein synthesis.

TABLE 1

The Growth Rate Constants K_0 and K Apparent
in Min^{-1} for Neomycin Affected *E. coli*,
A. aerogenes and *S. aureus* in Broth in 37.5°C .
and pH 7.00.

Drug Concentration $\mu\text{g ml}^{-1}$	<i>E. coli</i> ($\times 10^5$)	Slope <i>E. coli</i> ($\times 10^3$)
0	6.38	12.7 (K_0)
1	5.07	6.1 (K apparent)
10	4.48	5.7 "
100	4.32	3.8 "
500	3.41	3.0 "
1,000	3.32	2.3 "
10,000	3.22	2.7 "
<i>A. aerogenes</i>		
0	2.39	3.57 (K_0)
5	1.95	2.40 (K apparent)
50	1.60	1.23 "
500	1.40	1.70 "
<i>S. aureus</i>		
0	4.68	8.37 (K_0)
1	2.91	2.57 (K apparent)
10	2.78	2.13 "
100	2.59	1.50 "
500	3.45	4.37 "
1,000	3.19	3.50 "
10,000	3.30	3.87 "

From the data obtained it is quite apparent that the effect of neomycin on *E. coli* and *A. aerogenes*, both gram negative organisms, is directly related to the concentrations used.

However, the results obtained using *S. aureus*, a gram positive organism, were different. In this case as the drug concentration increased, a decreased inhibition was noted and also little difference, if any, was shown by using high or low neomycin concentrations once the minimum inhibitory concentration was reached. This could be attributed to the differences in cell structure of gram negative and gram positive organisms. Possibly at higher concentrations of neomycin the penetrability of the drug through the cell membrane is diminished which results in lesser inhibition.

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APPENDIX II

ANNUAL XAVIER-MBS BIOMEDICAL SYMPOSIUM

ABSTRACT FORM

FOR SESSION ON: (circle one) Biology, Biochemistry, Biophysics,
Chemistry, Medical Technology, Pharmacology,
Psychology, Other Microbiology

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USE OF COULTER COUNTER IN STUDYING EFFECTS OF DRUGS ON
CELLS IN CULTURES II. EFFECT OF CORDYCEPIN ON HE LA CELLS

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The application of coulter counter to study antimicrobial effects of drugs is now fairly well established. The technique has proven very useful in obtaining data for kinetic interpretation of the effects of antimicrobial drugs. In the present study the technique has been adapted to study the effects of cordycepin, a nucleoside antibiotic on He La cells (SH-503, International Scientific Industries). He La cells were incubated in the presence of cordycepin concentrations ranging from 0 to 500 μ g/ml for 5 days. It was noted that cordycepin at 100 μ g/ml incorporated at the start of the incubation period resulted in 76 per cent growth inhibition of cells. The growth inhibition due to cordycepin is cytostatic rather than cytotoxic. These results appear to be in agreement with other techniques, like colony formation in dilute agar used for determining cell viability.

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